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CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that the annexed is a true copy of the Provisional Specification as filed on 26 September 1997 with an application for Letters Patent number 328853 made by Auckland Uniservices Ltd.

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Dated 04 December 1998.

Neville Harris
Commissioner of Patents

Patents Form No. 4

Our Ref: DT209173

Patents Act 1953

PROVISIONAL SPECIFICATION

THERAPEUTIC METHOD

We, **AUCKLAND UNISERVICES LIMITED,** a New Zealand company, of 58 Symonds Street, Auckland, New Zealand do hereby declare this invention to be described in the following statement:

THERAPEUTIC METHOD

Technical Field

This invention relates to the use of amylin as an agent for stimulation of cartilage growth and hence its use in the treatment of cartilage disorders where stimulation of growth is required.

Background of Invention

Amylin is a 37-amino acid peptide cosecreted with insulin from the beta cells of the pancreatic islets. It was first reported by Cooper et al in Proceedings of the National Academy of Sciences USA 84, 8628 (1987) and is the subject of European Patent 289287. Amylin has the following peptide sequence:

Lys-Cys-Asn-Thr-Ala-Thr-Cys-Ala-Thr-Gln-
1 5 10

Arg-Leu-Ala-Asn-Phe-Leu-Val-His-Ser-Ser-
11 15 20

Asn-Asn-Phe-Gly-Ala-Ile-Leu-Ser-Ser-Thr-
21 25 20

Asn-Val-Gly-Ser-Asn-Thr-Tyr
31 35

The native molecule contains a disulfide bridge between the cysteine residues shown at positions 2 and 7 in the primary structure, is amidated at the 3'-end, and is formed as a propeptide.

European Patent 289287 reports a number of novel biological effects including enhancement of hepatic glucose output, increased production of lactate from skeletal muscle and reduced action of insulin in skeletal muscle.

Amylin is also reported in European Patent 408284 as having value for treatment of bone disorders and calcium imbalance. The patent specification attributes the activity of amylin to an inhibition of osteoclast motility. It is also reported in WO96/02269 as stimulating osteoblast proliferation.

Disclosure of the Invention

We have now found that administration of amylin stimulates cartilage growth. We believe this is the first report of the use of amylin causing increased proliferation of cartilage especially chondrocytes.

By the terms amylin or amylin agonist as used herein we mean amylin or any functionally effective derivative or fragment thereof or related peptide which binds to the amylin chondrocyte receptor and lead to amylin like effects. Fragments are described for example in EP 289287 which is herein incorporated by reference.

The invention therefore provides a method of stimulating cartilage growth comprising subjecting the cartilage to the effect of an effective amount of amylin or an amylin agonist.

The invention also provides a method of repairing damaged cartilage comprising subjecting the damaged cartilage to the effect of an effective amount of amylin or an amylin agonist.

The invention also provides a method of stimulating the growth of bone comprising subjecting cartilage, from which the bone grows, to the effect of an effective amount of amylin or an amylin agonist.

The invention further provides the use of amylin or an amylin agonist in the manufacture of a medicament for use in treating damaged cartilage or stimulating the growth of cartilage or bone.

Treatment of mammals can be with the homologous or heterologous amylin. Suitable amylin or amylin agonists can be those derived from animals, eg humans and other mammals e.g. rat, monkey, dog, cat, mouse, guinea pig, hamster, degus, rabbit, hare. The structure of these various peptides is reported in Endocrine Reviews 1994, 15 (2) 163 by Garth J S Cooper which is herein incorporated by reference.

Amylin and amylin agonists can be produced by methods well known in the art, eg as set forth in European Patent 289287, 309100 and 408284. Pure amylin is isolated by HPLC by these methods in the form of the trifluoroacetic acid salt (TFA salt) generally as the tetra-TFA salt.

These salts can be prepared in any known manner. For example, by simple reaction with the preferred acid eg. hydrochloric acid and then freeze drying. For example the salts for use in this invention can also be made by a method involving ion-exchange. This method may be carried out in either batch or column format. Reversed-phase chromatography will generally be the most effective and therefore the most useful. Other applicable forms of chromatography which can be used include size exclusion, ion-exchange, affinity and hydrophobic interaction.

A preparation of human amylin as the trifluoroacetate salt is dissolved in a solvent such as purified water, for example purified by double distillation or reverse osmosis coupled with filtration and ion exchange or another equivalent method or another suitable solvent, to which is added either a free acid, or salt thereof, particularly one which is soluble in the solvent. If necessary, the solution is buffered so that the pH lies between the range of 3.0 and 10.0, although preferably this will lie between 4.5 and 7.5. Buffering may be achieved either by addition to the aqueous solution of a base such as sodium hydroxide, potassium hydroxide or ammonium hydroxide alone, in which case reliance is placed upon the anion itself to

act as the buffering agent, or through the addition of well known buffers, including for example any of those described by Dawson RMC, Elliott DC, Elliott WH, Jones KM, in Data for Biological Research, Chapter 18, 3rd Edition, Oxford Science Publications, Oxford, 1986, pp 417-452. The final concentration of the free acid or acid anion may lie between 0.001% and 10% (wt/vol), preferably between 0.1 and 1.0 (wt/vol). The mixture is then stirred (for example using a magnetic stirrer) at a fixed temperature, preferably between 0°C and 37°C, for a suitable length of time which can be between 1 min and several days.

Acids or salts thereof which can be used in this process preferably include compatible inorganic acids such as hydrochloric and organic acids (or salts thereof) more preferably those occurring in living organisms, including but not limited to oxalic acid, glucuronic acid, pyruvic acid, lactic acid, citric acid, isocitric acid, α-ketoglutaric acid, succinic acid, malic acid, and oxaloacetic acid. In the preferred case of an aqueous solution, the desired anion can be added either as the free acid, or as a salt, preferably one which is highly soluble in water, for example the sodium or potassium salts, but also the lithium, magnesium, calcium or ammonium salts. Moreover, these salts can be used either in anhydrous or hydrated forms for example, citric acid can be used as the anhydrous free acid, the monohydrate free acid, the anhydrous trisodium salt, oir the dihydrate trisodium salt.

The aqueous solution of amylin containing the appropriate anion is then applied to a reverse phase resin, which can be contained in an open vessel or a column for example a SepPak column or a chromatography column. Reversed-phase resins suitable for this purpose include but are not limited to the following: (i) silica-based C1, C3, C4, C6, C8, C18, phenyl, and cyano forms, and non-silica-based forms including polyether- and polystyrene-based e.g. as described by Carron PH, Reversed-phase chromatography of proteins, in Oliver RWA (Ed) "HPLC of macromolecules: a practical approach", IRL Press, Oxford, 1989, pp 138-139. The reversed-phase resin is then washed with appropriate volumes, which may be between one and one hundred or more volumes equivalent of the resin, with a solution of the desired anion, either in the same form as that in

which the amylin preparation was dissolved, or any other suitable form. The amylin is then eluted by application of a solution of the anion in an aqueous solution of an organic solvent. Solvents which can be used for this purpose preferably include, but are not limited to acetonitrile, methanol, ethanol, propan-1-ol, propan-2-o1, and 2-methoxyethanol or mixtures thereof. The proportion of organic solvent in the aqueous phase can be fixed, for example, acetonitrile:water (70:30 (vol/vol)). Alternatively the solvent can be applied in the form of linear or stepped gradient, for example, elution can be performed by a solution of a solvent which varies in proportion between 0 and 100% (vol/vol) of the aqueous phase, such as an acetonitrile solution in water which varies between 0% and 100% (vol/vol). A preferred scheme for the elution of amylin salts other than trifluoroacetate salts from a reversed phase column is given in a modification of the method described by Cooper GJS, Willis AC, Clark A, Turner RC, Sim RB, Reid KBM, "Purification and characterization of a peptide from amyloid-rich pancreases of type II diabetic patients", Proc Natl Acad Sci USA 1987; 84: 8628-8632. The difference from that method being that trifluoracetic acid in the example is replaced by a salt with chosen anion or free acid.

Once the amylin is eluted from the reversed phase resin in this manner, it may be further purified, for example by dialysis. The chosen pure amylin salt is then obtained by removal of the solvent, for example by rotary distillation or by centrifugation under vacuum.

For use in therapy, amylin can be utilised by itself, a functionally effective derivative or as a fragment. Each class of substance has amine groups which can form salts in accordance with the invention. Amylin per se has four amino groups which therefore forms in the known production methods the tetratrifluoroacetic acid. It is believed that all the fragments which have amylin type activity, e.g. as set forth in European Patent 289287, will all have at least one amino group which will be capable of forming a salt. Where two or more amino groups are present in the substance, the possibility of mixed salts exists which are also within the scope of the invention.

Amylin can also be amidated at the C-terminal.

The compounds of the invention can be formulated into pharmaceutical compositions in the normal way to make oral, intranasal or parenteral formulations dependent upon the desired form of administration of the substance.

The amylin or amylin agonist is administered in an amount to meet the particular condition under treatment. It will be administered to the mammal either injectably, intranasally or in an oral formulation in a form for ensuring the availability of the amylin agent at the particular site for effective therapy. Amylin like most long chain peptides is generally inactive when taken orally. Fragments of amylin have been reported to have amylin like activity for other indications and it is anticipated that a fragment of amylin may have cartilage stimulation effects. Fragments which are small peptides may be able to be effective orally. Parenteral administration is therefore expected to be the most common employed either subcutaneously or intramuscularly. For the treatment of damaged cartilage, application of amylin directly to the site is desirable e.g. by injection or by application during surgery. It is believed that the dosage administered will lie within the range 0.01-100 mg/kg of body weight. The actual dose administered to each patient will depend on the type of patient and the nature of the disorder being treated.

Bone forms on a template of cartilage tissue and is the result of conversion of the cartilage to bone. Hence, one application of the administration of amylin or an amylin agonist is the stimulation of bone growth, in particular, the growth of limb bones.

Administration of two or more compounds selected from amylin or amylin agonists is within the scope of the invention as is the use of an amylin compound with any other effective therapeutic agent including any other agent for treatment of cartilage disorders. Combination agent therapy can be by separate administration of the individual agents or by combining the two or more agents into one composition form.

The following experimental section illustrates the invention.

METHODS

(a) Chondrocyte Cell Cultures

Fresh cartilage samples were collected from the tibial plateaus and femoral condyles of mature, healthy crossbred dogs (2-4 years; 20-25 kg). The chondrocytes were isolated as previously described (Connective Tissue Research 1988;18:205-222). Briefly, the chondrocytes were obtained by pronase and collagenase digestion of the cartilage, then the cells were centrifuged, washed and resuspended in media before being cultured in 75 cm^2 tissue culture flasks. The cells were incubated under 5% CO_2 and 95% air at 37°C. Confluence was reached by 7-10 days, at which time the cells were subcultured. After trypsinization, the cells are rinsed and resuspended in fresh medium, then seeded at 5 x 10^4 cells/ml in 24-well plates (0.5 ml cell suspension per well, ie. 1.4 x 10^4 cells/cm^2). *Proliferation studies* (cell counts and thymidine incorporation) were performed. Subconfluent population were changed to serum-free medium with 0.1% bovine serum albumin plus the experimental compounds. Cell numbers were analysed at 24 hours after addition of the peptide or vehicle. The cell numbers were determined using a haemocytometer chamber. Results were expressed per well. [^{3}H]-*thymidine incorporation* was assessed by pulsing the cells with [^{3}H]-thymidine (1uCi/well) two hours before the end of the experimental incubation. The experiment was terminated at 24 hours by washing the cells in media containing cold thymidine followed by the addition of 10% trichloroacetic acid. The precipitate was washed twice with ethanol:ether (3:1) and the wells desiccated at room temperature. The residue was redissolved in 2 M KOH at 55°C for 30 mins, neutralized with 1 M HCl, and an aliquot counted for radioactivity. Results were expressed as dpm per well. For both cell counts and thymidine incorporation, each experiment was performed at least 4 different times using experimental groups consisting of at least 6 wells.

(b) In Vivo Study: Experimental Design

Two groups of 20 sexually mature male Swiss mice aged between 40 and 50 days and weighing 25-32g, were given daily subcutaneous injections (50 ul) in the loose skin at the nape of the neck for 5 days/week over 4 consecutive weeks. The amylin treated group was injected with rat amylin at a dose of 300ug/kg/injection and the control group was injected with vehicle (water). Animals were housed in a room maintained at 20°C on 12-hour light/dark cycles. They were fed diet 86 rodent pellets (New Zealand Stockfeed Ltd) ad libitum throughout the experiment. Each animal's weight was recorded at the beginning and end of the experiment. One day after the last injection, animals were sacrificed by cervical dislocation. The study had the approval of the local institutional review board.

The tibiae were dissected free of adherent tissue. Tibial lengths were recorded by measuring the distance between the proximal epiphysis and the distal tibio-fibular junction using an electronic micrometer (Digimatic Calipers, Mitutoyo, Japan). Bones were placed in 10% phosphate-buffered formalin for 24 hours and then dehydrated in a graded series of ethanol solutions and embedded, undecalcified, in methylmethacrylate resin. Tibiae were sectioned longitudinally through the frontal plane and calvariae were cut cross-sectionally at the base of the parietal bone. All sections were 4 um thick and were cut on a Leitz microtome using a tungsten-carbide knife (Microknife Sharpening, Utah, USA). Sections were mounted on gelatin-coated slides and air-dried. They were stained with Goldner's tri-chrome and examined using an Olympus BX 50 microscope (Olympus Optical Co Ltd, Tokyo, Japan) which was attached to an Osteomeasure Image Analyzer (Osteometrics Inc, Atlanta, GA).

Tibial histomorphometric analyses were made from three adjacent sections one third of the way through the anterior/prosterior depth of the proximal tibiae. Epiphyseal growth plate thickness was measured at three sites evenly spaced along its length. All measurements were made by one operator who was blinded to the treatment group of each bone.

Materials

Rat amylin was sourced from Bachem California, Torrance, CA, USA. Lyophilised material was dissolved in water prior to administration. Methylmethacrylate was purchased from Acros Organics N.V., Geel, Belgium.

Statistical Analysis

Data are presented as mean ± sem. Where parameters have been measured more than once in each animal these values have been averaged to produce a single value for each animal before further analysis. The significance of treatment effects was evaluated using Student's *t* tests for unpaired data. These comparisons were specified a priori, so adjustment of α(0.05) was not necessary.

RESULTS

(a) Chondrocyte Cell Cultures

Amylin influenced chondrocyte proliferation, increasing cell numbers from 4.12 ± 0.23 ($x10^4$) (mean ± sem) in control cells to 5.11 ± 0.21 ($x10^4$) in those cells incubated with amylin ($p=0.01$) as well as increasing thymidine incorporation (ie, DNA synthesis) from 20725 ± 997 dpm in control cells to 25937 ± 1203 dpm in amylin-treated cells.

(b) In Vivo Study

Amylin influenced the tibial growth plate, increasing its width from 0.083 ± 0.005 mm (mean ± sem) in the control animals to 0.108 ± 0.003 mm in those receiving amylin ($P = 0.0002$) (Figure 1). The total length of the tibiae was also increased from 11.31 ± 0.07 mm in control animals to 11.67 ± 0.09 mm in animals injected with amylin (P - 0.004) (Figure 2).

Discussion

The effects of amylin in vivo on the width of the growth plate and on tibial length imply that the chondrocyte is also an amylin-target cell. These changes in chondrocyte proliferation in response to amylin are comparable in magnitude to those we have previously described in osteoblasts with this peptide. The present findings suggest that hyperamylinemia may be associated with increased linear growth. Since nutrient intake results in amylin secretion, the regulation of chondrocyte function by amylin provides a pathway by which growth and the availability of the substances for growth from food might be linked. These findings further suggest that amylin and related peptides (eg adrenomedullin) may be able to be used therapeutically for promoting linear growth, for example in children with short stature, whether naturally or as a result of the number of medical conditions that have been identified as causing this problem. In addition these results indicate that amylin could be usefully employed in the treatment of damaged cartilage.

Legends To Figures

Figure 1: Effects of daily systemic administration of amylin for 4 weeks on growth plate width in the tibiae of normal adult male mice. n = 20 in each group. *, significantly different from control,
P = 0.0002

Figure 2: Effects of daily systemic administration of amylin for 4 weeks on bone length in the tibiae of normal adult male mice.
n = 20 in each group. *, significantly different from control,
P = 0.004.

AUCKLAND UNISERVICES LIMITED
By Its Attorneys
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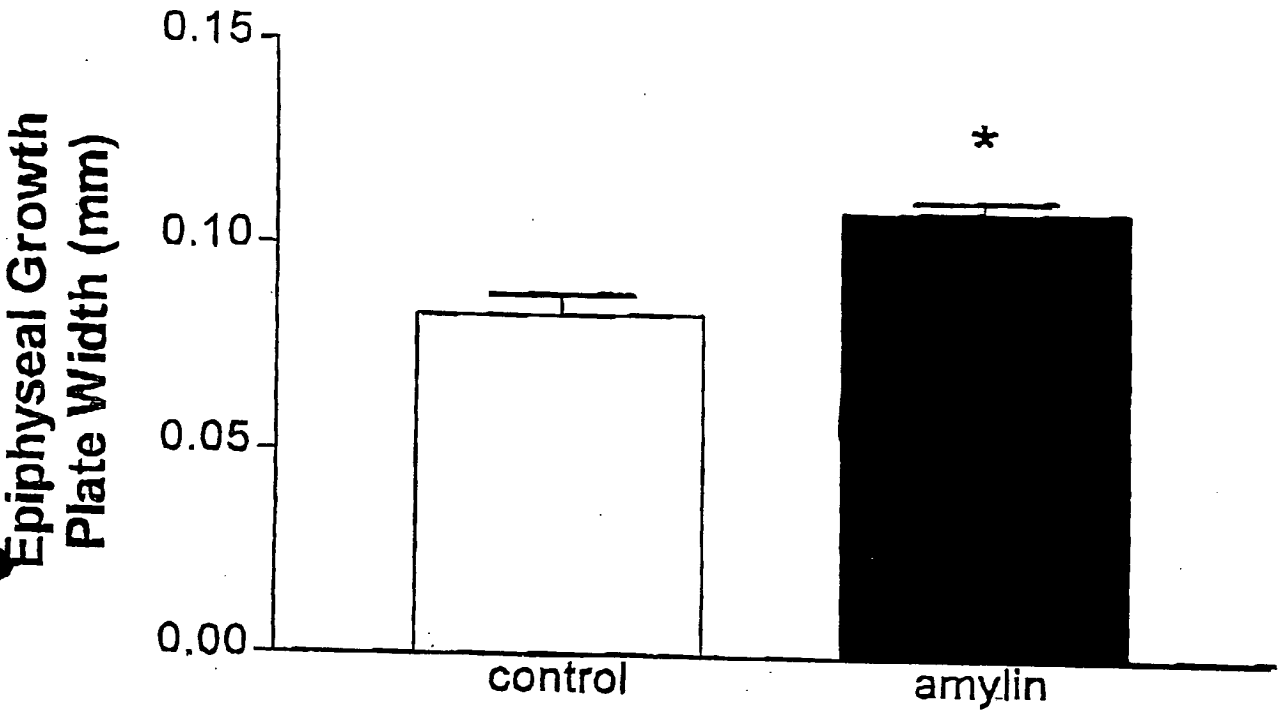

FIGURE 1

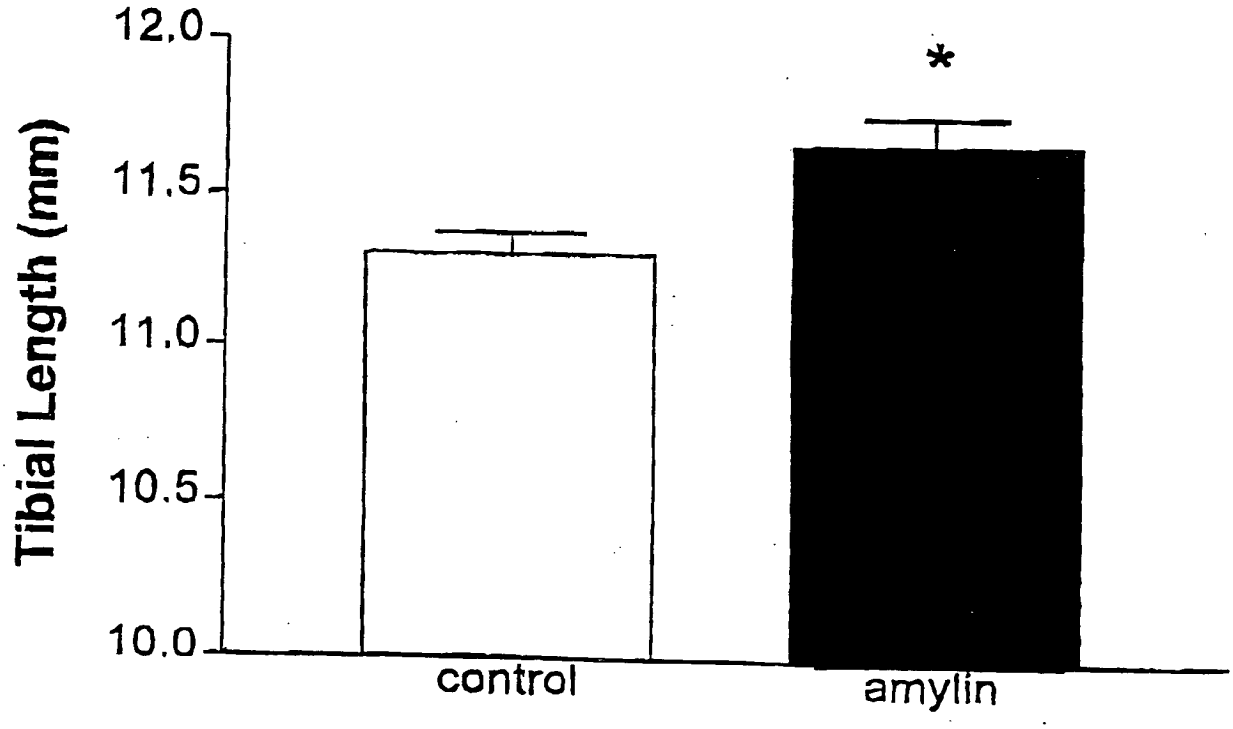

FIGURE 2